

## Recircularization and Autonomous Replication of a Sheared R-Factor DNA Segment in *Escherichia coli* Transformants\*

(plasmid/transformation/antibiotic resistance/DNA)

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**ABSTRACT** Controlled shearing of R-factor DNA leads to formation of fragments carrying an antibiotic resistance gene present on, but not expressed by, the intact R-factor. Transformation of  $\text{CaCl}_2$ -treated *E. coli* by such fragments yields an autonomously replicating tetracycline-resistance plasmid (Tc6-5) that contains only a small fraction of the genome of the parent R-factor, and lacks both its fertility functions and its other drug-resistance determinants. Although the Tc6-5 plasmid is not self-transmissible, it can interact and/or recombine with conjugally-proficient plasmids that promote its transfer to other bacteria.

R6-5 is a spontaneous mutant of the R-factor R6 that has lost the tetracycline resistance expressed by the parent R-factor (1). Earlier studies (2) utilizing electron microscope heteroduplex techniques (3) have indicated that the tetracycline sensitivity of R6-5 results from insertion of a plasmid DNA nucleotide sequence in *reverse sense* adjacent to the R6 DNA region specifying resistance to this antibiotic. The DNA insertion, which prevents expression of the tetracycline-resistance genes still carried by the plasmid, is spontaneously excised at very low frequency leading to regeneration of a plasmid that is phenotypically and structurally identical to the tetracycline-resistant parent R6 (2).

We have reported (4) that calcium chloride-treated *Escherichia coli* can be genetically transformed to antibiotic resistance by use of purified R-factor DNA, and that the transformed bacteria acquire a closed-circular DNA species that has the resistance, fertility, and sedimentation characteristics of the parent R-factor. Whereas covalently closed, nicked circular, and catenated forms of R-factor DNA are all effective in transformation, denaturation or sonication of the DNA destroys its ability to transform.

The present report describes experiments involving controlled shearing of DNA of the R-factors R6-5 and R6, and subsequent transformation of *E. coli* by the sheared plasmid DNA. Our results indicate that: (i) a DNA segment carrying genetic information for tetracycline resistance, but lacking the other antibiotic resistance determinants of the R-factor, is produced from both R6 and R6-5 by appropriate shearing; (ii) the R-factor DNA fragment produced by shearing recircularizes in transformants, replicates autonomously as a plasmid, and is recoverable from transformed bacteria as a covalently closed-circular DNA species; and (iii) the newly

formed plasmid, which contains less than 10% of the parent R-factor genome, is not self-transmissible, but can be conjugally transmitted to other bacteria by a naturally occurring R-factor transfer unit or by *F-lac*. In addition, it can recombine covalently with a related R-factor to give rise to a new transferable antibiotic-resistance plasmid.

### MATERIALS AND METHODS

**Bacterial Strains and R-factors.** Most bacterial strains and R-factors used in these experiments have been described (4). CR34N is a spontaneous mutant of *E. coli* strain CR34 that has been selected (5) for chromosomal resistance to nalidixic acid (100  $\mu\text{g}/\text{ml}$ ). SP (I) is a strain of *Salmonella panama* carrying the naturally occurring *fi*<sup>-</sup> transfer unit I (7).

**DNA Preparations.** Covalently closed DNA preparations were isolated and purified from *E. coli* as indicated (4). Controlled shearing of DNA (8) was accomplished with a Fisher steady-speed stirrer fitted with a Virtis stainless steel micro-shaft, blade, and 1.0-ml cup. Samples were sheared at 2000 rpm for the indicated period, and were chilled at 0–4° in ice water during shearing. Sucrose gradient centrifugation of DNA and analytical CsCl sedimentation equilibrium centrifugation were performed as described (4, 5). Circular plasmid DNA was treated with Sarkosyl, Pronase, or pancreatic deoxyribonuclease as described by Clewell and Helinski (13).

**Electron Microscopy.** Specimens were prepared by modifications of the Kleinschmidt technique (9) described by Hudson and Vinograd (10), and were examined on a Philips EM-300 electron microscope (5).

**Transformation.** Transformation of *E. coli* by R-factor DNA was performed as described by Cohen, Chang, and Hsu (4), except that bacterial cells were grown to  $A_{590} = 0.600$  and were incubated with R-factor DNA for 30 min at 0° before being subjected to a heat pulse at 42°.

### RESULTS

Sucrose gradient sedimentation patterns of a sample of R6-5 DNA that has been subjected to shearing for different periods of time are shown in Fig. 1. The resistance patterns observed in *E. coli* strain C600 after transformation by sheared or intact R6-5 DNA are indicated in Table 1A. As seen in Fig. 1, the unsheared DNA sample consisted mostly of covalently closed-circular molecules of DNA sedimenting at 75.5 S that has similar sedimentation properties to the DNA of the parent

Abbreviation: R-factor, antibiotic-resistance factor.

\* The previous paper in this series, *Nonchromosomal Antibiotic Resistance in Bacteria*, is ref. 4.

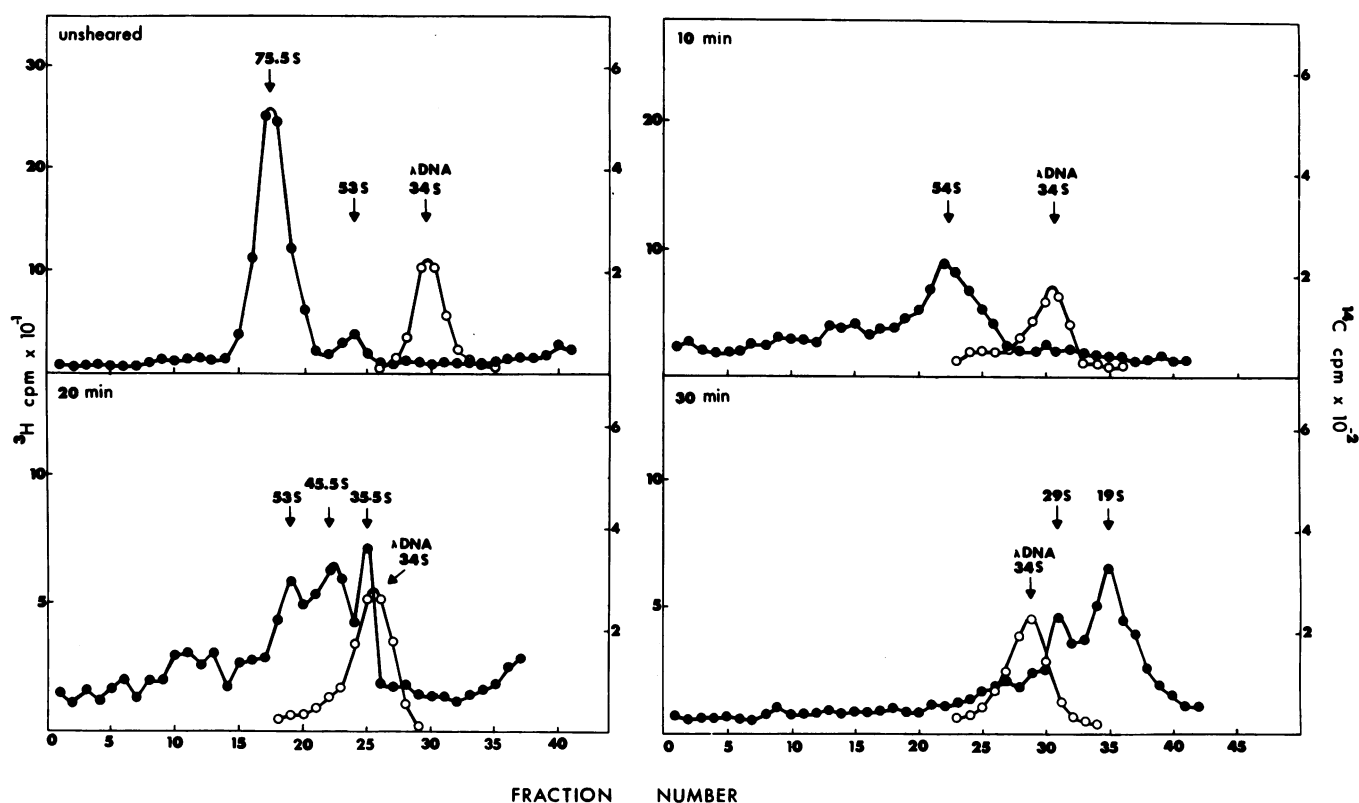


FIG. 1. Sucrose gradient sedimentation patterns of sheared and intact R6-5 DNA. Sedimentation was at 39,500 rpm for 60 min (un-sheared) or 75 min (sheared) in a Spinco SW 50.1 rotor at 20° in the presence of a  $\lambda$ [<sup>14</sup>C]DNA (34 S) marker. 0.12-ml fractions were collected on 2.3-cm diameter circles of Whatman no. 3 filter paper, dried for 20 min under an infrared lamp, and precipitated by immersion of discs in cold 5% trichloroacetic acid containing thymidine (100  $\mu$ g/ml). The filters were washed once with cold 5% Cl<sub>3</sub>CCOOH, and twice with 99% ethanol, air dried, and counted (5). The times indicated refer to the duration of shearing in this experiment. In other experiments, a 25–80% increase in the duration of shearing was required in order to produce equivalent fragmentation of R6-5 DNA. Frequently, this fragmentation pattern could not be produced by shearing in any single experiment.

TABLE 1. Effect of shearing on transformation by R-factor DNA

R-factor DNA	Transformation frequency for resistance markers ( $\times 10^7$ )				
	Tc	Cm	Km	Nm	Sm
(A) R6-5					
Unsheared	0.11	50	50	50	0.05
Sheared 10 min	*	5	3	3	0.05
Sheared 20 min	*	2.5	3	3	*
Sheared 30 min	0.25	*	*	*	*
(B) R6					
Unsheared	50	50	50	50	0.3
Sheared 30 min	0.47	*	*	*	*

0.1- $\mu$ g Samples of the same DNA preparations studied in Fig. 1 were used for transformations. Antibiotic concentrations used for selection of transformants were as in ref. 4. The transformation frequency is expressed as the number of antibiotic-resistant bacteria relative to the total number of viable cells. Although transformation frequency was low when transformants were selected for Sm resistance, nearly all transformants selected for the Cm, Km, or Nm markers also expressed Sm resistance, as reported (4).

\*  $< 2.5 \times 10^{-9}$ , which was the minimum level of detection of transformation frequency in these experiments. Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Nm, neomycin.

R-factor, R6 (5). A small amount of open-circular DNA sedimenting at 53 S was also seen, as has been described for R6 (5). Unsheared plasmid DNA isolated from tetracycline-sensitive bacteria carrying R6-5 yielded tetracycline resistant transformants (Table 1A) that were phenotypically identical to the rare (frequency, about  $10^{-9}$ ), tetracycline resistant spontaneous revertants of R6-5 that have been observed (2). After 10 min of shearing, most molecules in this DNA preparation were converted to the 54.5S (open-circular) form of R6-5. Shearing of R6-5 DNA for this length of time was associated with an overall reduction in its transforming ability, but did not affect the antibiotic resistance pattern of transformants (Table 1A), which continued to express all of the resistance determinants carried by R6-5. Shearing of DNA for 20 min led to the appearance of 44.5S and 35.5S DNA species in addition to the 53S molecules, but also did not change the drug-resistance pattern of transformants.

Shearing of R6-5 DNA for 30 min resulted in the production of 27S and 19S fragments of the R-factor. As seen in Table 1A, this sheared DNA preparation yielded *only tetracycline-resistant* transformants that lacked all of the other drug-resistance markers of R6-5. Thus, genetic information specifying tetracycline resistance could be recovered from the DNA of a tetracycline-sensitive R-factor by mechanical shearing of this DNA, and subsequent transformation with the R-factor DNA fragments. As shown in Table 1B, shearing of R6 DNA for 30 min also resulted in the production of transformants

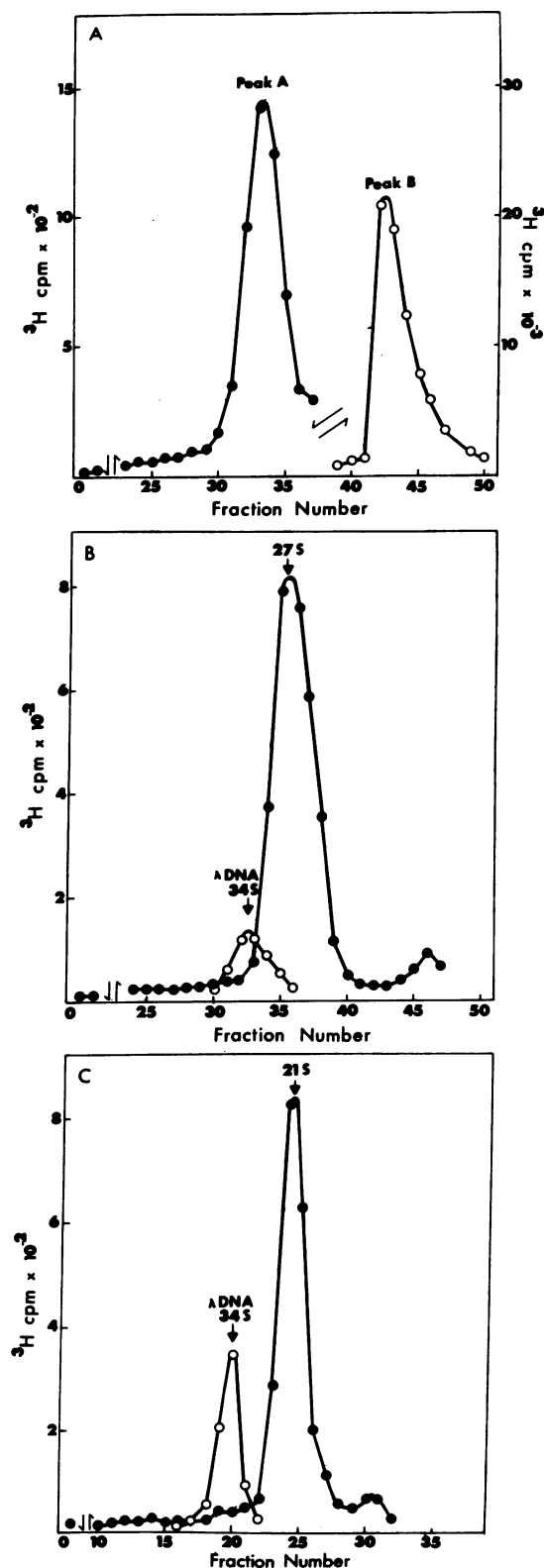


Fig. 2. Centrifugation analysis of DNA present in tetracycline-resistant transformed cells. (A)  $[^3\text{H}]\text{DNA}$  was prepared and isolated (4, 6) and was centrifuged for 44 hr at 39,500 rpm at  $20^\circ$  in  $\text{CsCl}$  ( $\rho = 1.557$ ) containing ethidium bromide (800  $\mu\text{g}/\text{ml}$ ) in a Spinco 50 Ti rotor. Collection and assay of fractions for radioactivity has been described (5). (B) After removal of ethidium bromide (11) from fractions 32–35 of peak A, DNA was dialyzed against 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–20 mM

that carried tetracycline resistance, but that had none of the other drug-resistance determinants expressed by the intact R-factor.

#### Molecular nature of DNA isolated from Tc-resistant transformants

DNA isolated from a transformed *E. coli* clone carrying tetracycline resistance was centrifuged through cesium chloride-ethidium bromide gradients. As seen in Fig. 2A, a closed-circular DNA peak (peak A) was identified in this preparation, in addition to a peak (peak B) characteristic of DNA preparations isolated from the  $\text{R}^-$  *E. coli* recipient strain, C600. R-factor DNA collected from peak A was treated with isopropanol to remove ethidium bromide (11) and dialyzed (4); a sample of this DNA was centrifuged in a 5–20% neutral sucrose gradient in the presence of a 34S DNA marker prepared from bacteriophage  $\lambda$  (5). As seen in Fig. 2B, the circular DNA peak isolated from a  $\text{CsCl}$ -ethidium bromide gradient consisted of a 27S species having a calculated (12) molecular weight of  $5.8 \times 10^6$ . The sedimentation properties of this DNA species were not altered by treatment with Sarkosyl (0.2%) or incubation for 30 min with Pronase (1.5 mg/ml) (13), suggesting that it did not contain "relaxation complex" molecules. However, incubation with pancreatic DNase [ $2.5 \times 10^{-4}$   $\mu\text{g}/\text{ml}$ ] (13) resulted in complete conversion of the 27S closed-circular DNA to a 21S open-circular form (Fig. 2C).

Electron microscopy of the freshly prepared 27S DNA revealed only tightly coiled helical molecules that were unsuitable for contour length measurements. Examination of nuclease-treated preparations showed open-circular molecules having an approximate contour length of 3  $\mu\text{m}$  (Fig. 3), consistent (14) with the molecular weight of  $5.8 \times 10^6$  calculated from the observed 27S value in sucrose gradients.

Transformation of *E. coli* strain C600 with 27S DNA of the tetracycline-resistance plasmid (named Tc6-5) formed by a sheared fragment of R6-5 DNA occurred at a frequency ( $1.2 \times 10^6$  transformants per  $\mu\text{g}$  of DNA) similar to that observed for unsheared R6 DNA (4), whereas transformation for tetracycline resistance by R6 DNA that had been subjected to shearing for 30 min was at least 100–1000 times lower (Table 1B).

Analytical sedimentation equilibrium centrifugation of a sample of DNA from peak A of Fig. 2 is shown in Fig. 4. As seen in this figure, Tc6-5 DNA has a buoyant density of 1.710  $\text{g}/\text{cm}^3$ , which is similar to the buoyant density of the transfer unit segment of the parent R-factor (5), and is consistent with genetic (15) and electron microscopic (2) evidence indicating that the DNA region specifying tetracycline resistance in R6 is located in close proximity to genes controlling transfer of the R-factor.

#### Interbacterial transfer of the Tc-resistance plasmid

Tc6-5 was not self-transmissible by conjugation (Table 2, line 3), but could be mobilized (16) to other bacteria from an

$\text{NaCl}$ , and a 0.1-ml aliquot was layered onto a 5–20% linear sucrose gradient (4) and centrifuged for 90 min at 39,500 rpm in a Spinco SW 50.1 rotor. Fractions were collected and assayed as indicated in Fig. 1. (C) This DNA sample was treated as indicated for B, except that it was incubated at  $24^\circ$  with pancreatic DNase (250  $\mu\text{g}/\text{ml}$ ) for 20 min in the presence of 10 mM  $\text{MgCl}_2$  before it was layered onto a 5–20% sucrose gradient.

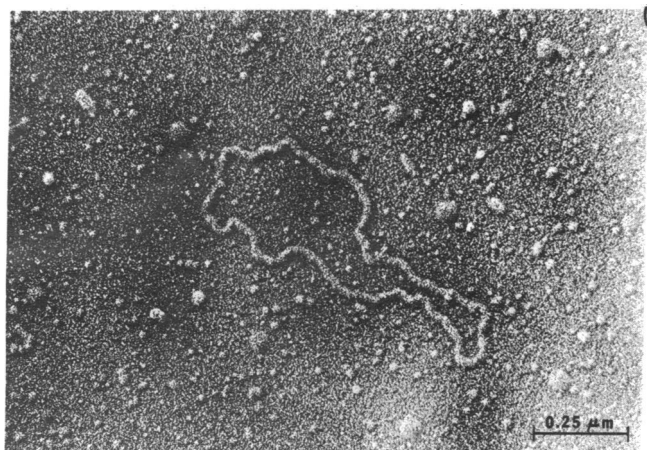


FIG. 3. Electron photomicrograph of an open-circular molecule of tetracycline-resistance plasmid derived from sheared R6-5 DNA. The approximate contour length of this molecule is 3  $\mu$ m.

intermediate recipient [C600 (Tc6-5)] by I (Table 2, line 4), a naturally occurring transfer unit isolated originally from *Salmonella panama*. Tc6-5 was also transferred from cells carrying this plasmid plus either the related (2)  $fi^+$  R-factor R1 or *F-lac* (Table 2 lines 7 and 9), but was not transferred by the isolated transfer unit (17) of R1 (line 8) at a detectable frequency. Our failure to observe mobilization of Tc6-5 by R1 or *F-lac* in a three-factor mating may simply reflect the comparatively low efficiency of this assay (see mobilization by transfer unit I); as seen in Table 2, when Tc6-5 is introduced by transformation into cells containing R1 or *F-lac*, transfer is accomplished by either of these plasmids.

After mobilization of Tc6-5 to a recipient strain by SPI, and subsequent isolation of closed-circular DNA from recipient cells by CsCl-ethidium bromide gradient centrifugation, Tc6-5 is seen to exist as a separate 27S DNA species (Fig. 5A). Closed-circular (65 S) and nicked-circular (44 S) forms of I DNA are also observed in the recipient cells. Moreover, the buoyant density of Tc6-5 is unchanged (Cohen and Chang, unpublished data) in the final recipient after its

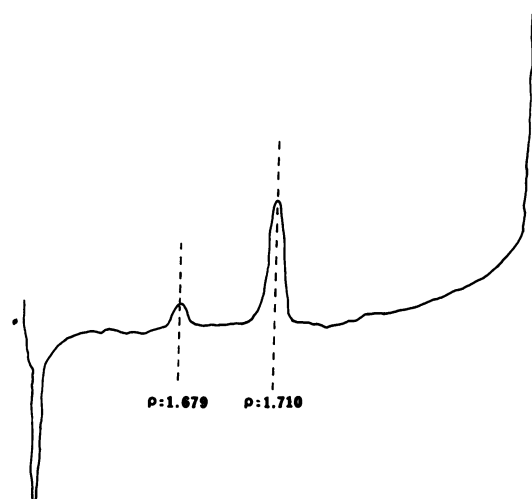


FIG. 4. Analytical ultracentrifugation of DNA obtained from peak A of Fig. 2A. Centrifugation in CsCl ( $\rho = 1.710$  g/cm<sup>3</sup>) (5) was for 21 hr at 44,000 rpm in the presence of a (dA-T)<sub>n</sub> ( $\rho = 1.679$  g/cm<sup>3</sup>) density marker. A densitometer (Gilford) tracing of a photograph taken during centrifugation is shown.

mobilization by I ( $\rho = 1.702$  g/cm<sup>3</sup>). In contrast, transfer of Tc6-5 by the 75S R1 is associated with covalent linkage of the two plasmids and formation of a recombinant, self-transmissible R-factor that sediments at 43 S (Fig. 5B). The recombinant plasmid has apparently undergone deletion of certain R1 DNA sequences, but now carries both tetracycline-resistance and drug-resistance determinants of R1.

## DISCUSSION

These experiments indicate that segments of sheared R-factor DNA containing less than 10% of the total R-factor genome (molecular weight,  $65 \times 10^6$ ) are capable of transforming CaCl<sub>2</sub>-treated *E. coli* to antibiotic resistance. Moreover, they demonstrate that sheared R-factor DNA segments (or fractional components of sheared segments) can recircularize in transformed bacteria, and can be maintained as autonomously replicating but, not self-transmissible DNA units that are transferable to other bacteria by interaction and/or recombination with conjugally-proficient plasmids. Sharp, Cohen, and Davidson (2) have shown by electron microscope heteroduplex techniques that expression of the tetracycline-

TABLE 2. Conjugal transfer of tetracycline resistance

	Frequency of transfer of tetracycline resistance
(A)	
1. C600 (R6-5) x CR34N	<10 <sup>-9</sup>
2. C600 (R6) x CR34N	$6 \times 10^{-6}$
3. C600 (Tc6-5) x CR34N	<10 <sup>-9</sup>
4. SP(I) x C600 (Tc6-5) x CR34N	$1.3 \times 10^{-6}$
5. C600 (R1) x C600 (Tc6-5) x CR34N	<10 <sup>-9</sup>
6. C600 (RTF1) x C600 (Tc6-5) x CR34N	<10 <sup>-9</sup>
(B)	
7. C600 (R1)(Tc6-5) x CR34N	*
8. C600 (RTF1) (Tc6-5) x CR34N	<10 <sup>-9</sup>
9. C600 ( <i>F-lac</i> ) (Tc6-5) x CR34N	†

(A) The frequency of transfer of tetracycline resistance in direct conjugal mating (lines 1-3) was determined (1). The procedure of Anderson and Lewis (16) was used (lines 4-6) to mobilize tetracycline resistance from an intermediate recipient. In all instances, tetracycline (5  $\mu$ g/ml) was used for selection of final recipient bacteria, which were then tested for resistance to 25  $\mu$ g/ml of this antibiotic. Nalidixic acid (100  $\mu$ g/ml) was present in all selective media.

(B) Cells carrying R1, RTF1, or *F-lac* were transformed by Tc6-5 DNA, and the frequency of transfer of the tetracycline-resistance determinant from tetracycline-resistant transformants was determined as in A.

\* In the experiment shown, detectable transfer of tetracycline resistance to CR34N was observed with only 1 of 24 selected tetracycline-resistant transformants of C600 (R1), at a frequency of  $1.7 \times 10^{-9}$ .

† 13 of 24 selected tetracycline-resistant transformants of C600 (*F-lac*) could transfer this resistance to CR34N (frequency of transfer, 1 to  $3 \times 10^{-9}$ ). Retesting of isolates from initially negative transformants often gave positive results for transfer, indicating that the initial failure to observe transfer from some colonies probably reflects a low frequency of transfer from a given culture, rather than genetic differences among the transformants. The antibiotic resistance markers expressed by R1 are ampicillin, Cm, Km, Sm, and Su (sulfonamide).

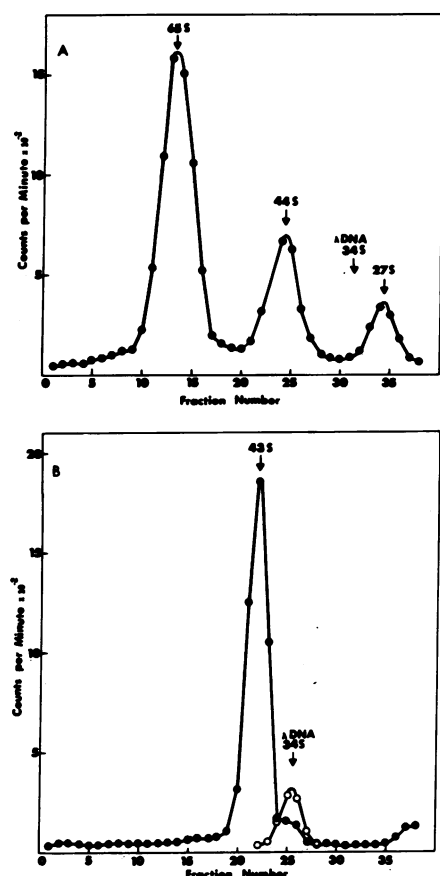


FIG. 5. Sucrose gradient sedimentation of plasmid DNA isolated from *E. coli* strain CR34N after transfer of tetracycline resistance to the strain by I or R1. Centrifugation was (A) for 90 min or (B) for 75 min. The position of 34S DNA from bacteriophage  $\lambda$  is indicated in both gradients.

resistance genes carried by R6-5 is prevented by insertion of a "reverse repeat" of a gene sequence adjacent to the region specifying tetracycline resistance. The present studies provide independent confirmation of the earlier electron microscope findings indicating that R6-5 carries tetracycline-resistance genes that it fails to express, since the Tc6-5 plasmid derived from the parent R6-5 specifies this resistance marker. Moreover, the observed molecular size of Tc6-5 and its capacity for autonomous replication indicate that this plasmid carries several other genes present on the parent plasmid in addition to the gene(s) coding for tetracycline resistance.

Our results indicate that the genes required for plasmid replication and an initiator site for such replication are present on the Tc6-5 plasmid. Presumably, the replication machinery for the parent R-factor is located near the site of tetracycline resistance, since autonomously replicating small plasmids that singly carry any of the other drug-resistance markers present on the intact R-factor were not observed after shearing.

R-factors have been classified on the basis of their ability to inhibit the fertility of F-factors into  $fi^+$  (fertility inhibition<sup>+</sup>) and  $fi^-$  groups (18). Genetic (18, 19), DNA-DNA

hybridization (20), and electron microscope heteroduplex (2) experiments indicate that  $fi^+$  R-factors generally differ greatly from  $fi^-$  R-factors in DNA nucleotide sequence and phenotypic characteristics. Thus, it is of interest that an  $fi^-$  transfer unit can mobilize a segment of the  $fi^+$  R-factor R6 that is not self-transmissible. The implications of this finding for the formation and evolution of R-factors warrant further consideration.

These experiments also suggest that the distinction between Class 1 (21) [or "co-integrate" (22)] R-factors (in which the transfer and resistance determinant units are covalently linked in *E. coli*) and Class 2 (21) [or "aggregate" (22)] R-factors (in which the two components of the plasmid remain separate in *E. coli*) is apparently determined by the nature of the transfer unit. Although it was derived from a Class 1 R-factor, Tc6-5 shows the phenotypic characteristics of a resistance-determinant plasmid of the Class 2 type, and maintains its identity as a discrete unit when mobilized by transfer unit I (Fig. 5A). However, it recombines covalently with the related Class 1 R-factor, R1 (Fig. 5B). Further studies of molecular and structural interactions between Tc6-5 and other bacterial plasmids will be published elsewhere (Cohen and Chang, in preparation).

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